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Award Number: DAMD17-99-1-9493

TITLE: Pro-apoptotic Changes in Brain Mitochondria After Toxin Exposure

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REPORT DATE: July 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | July 2002 | Annual (1 Jul 01 - 30 Jun 02) |

**4. TITLE AND SUBTITLE**
Pro-apoptotic Changes in Brain Mitochondria After Toxin Exposure

**5. FUNDING NUMBERS**
DAMD17-99-1-9493

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**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** ***(Maximum 200 Words)***

Mitochondria normally function to provide sources of energy for vital cellular functions. However, under stressful conditions these organelles may trigger events that lead eventually to cell death. Thus, mitochondria have been implicated as major contributors to neuronal death in a variety of neurodegenerative disorders. In this report we provide evidence that certain mitochondrial toxins cause selective cell death in hippocampal subfield CA1 that has previously been shown to be selectively vulnerable to hypoxia/ischemia. We have also measured changes in mitochondrial membrane potential following toxin exposure. Respiratory chain Complex I inhibitors caused mitochondrial depolarization by the degree of depolarization, although significant, was not dramatic. No mitochondrial depolarization was observed after excitotoxin or Complex II inhibition. All toxins tested produced an increase in reactive oxygen species. Our data show no evidence for mitochondrial permeability transition after toxin exposure.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| Neurotoxin | 37 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

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Tony Lau
Thomas T. Lau 7-30-02
PI - Signature Date

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## Introduction

It has long been thought that environmental factors contribute to the neurodegeneration characteristic of many neurological diseases. In the past decade, considerable attention has been directed toward environmental agents that inhibit mitochondrial function. This work has provided evidence that mitochondria provide signals to cells, including neurons, that initiate activation of processes leading to programmed or "apoptotic" cell death. The goals of this contract are: 1) to first characterize toxin-induced alterations of respiratory chain redox status and mitochondrial membrane potential in intact functioning neuronal populations; 2) to examine the role of specific intracellular mediators of mitochondrial and neuronal dysfunction including release of mitochondrial cytochrome c, calcium overload, reactive oxygen species (ROS), and mitochondrial permeability transition. Experiments were to be conducted in intact functionng brain slices preparations using optical methodology to monitor changes in mitochondrial function, and electrophysiological methods to monitor neuronal function.

## Body

In the first year of this contract, experiments were conducted to establish baseline responses of mitochondrial redox activity and hippocampal electrophysiology to toxin exposure. We also established dose response relationships for the putative Parkinson's disease toxin, 1-methyl-4-phenylpyridium ($MPP^+$), the excitotoxin N-methyl-D-aspartate (NMDA), and 3-nitropropionic acid (3-NP), a mitochondrial toxin used to model Huntington's disease. In the second year of the contract we established an organotypic slice culture model to directly assess the effects of toxin exposure on neuronal cell death, and we began studies of rotenone, a mitochondrial Complex I inhibitor that had been shown to induced Parkinson's-like neuropathology in experimental animals. In this report, we present data that concludes some of these earlier studies. We also show new data describing the effects of the toxins on mitochondrial ROS production in hippocampal slices. Finally, we address the hypothesis that mitochondrial permeability transition plays a role in toxin-induced neuronal damage.

### Delayed Hippocampal Cell Death After Toxin Exposure

In these experiments, organotypic hippocampal slice cultures were grown for 2-3 weeks in culture and then exposed for 1 hr to toxins. Cell death was examined 24 hr, 48 hr and 72 hr later using the fluorescent dye propidium iodide, that only stains cells with damaged plasma and nuclear membranes. In our previous report, we provided preliminary data that mitochondrial Complex I inhibitors could cause selective neuronal cell death in hippocampal subfield CA1 compared to subfield CA3. In this respect, it appeared that inhibition of Complex I with mitochondrial toxins produced damage similar to that observed in hypoxia/ischemia. Such damage had not been reported earlier in studies that examined damage to the striatum and substantia nigra following exposure of experimental animals to these toxins. In this report, we present the conclusion of this study in manuscript form (Appendix). We found that the respiratory chain Complex I inhibitor rotenone, and to a lesser extent $MPP^+$, selectively damaged neurons in

hippocampal subfield CA1. The Complex II inhibitor, 3-NP, produced little neurotoxicity, and the excitotoxin NMDA caused damage to both the CA1 and CA3 subfields.

Mitochondrial Permeability Transition After Toxin Exposure

Mitochonddrial permeability transition (MPT) is characterized by a sudden increase in permeability of the inner mitochondrial membrane, collapse of the mitochondrial membrane potential, and release of pro-apoptotic signaling molecules such as cytochrome c. MPT has been shown to occur under a variety of conditions although mainly in cell culture and in isolated mitochondrial preparations. There have been a few reports that MPT may occur after ischemia in the intact brain but these studies have not been conclusive. A major goal of this contract was to determine if there is evidence of MPT following toxin exposure.

Mitochondrial Membrane Potential – A key feature of MPT is collapse of the mitochondrial membrane potential. We measured changes in mitochondrial membrane potential in hippocampal slices using the fluorescent indicator JC-1. JC-1 accumulates in mitochondria in proportion to the mitochondrial membrane potential. At high concentrations the dye shows red fluorescence while in low concentrations its fluorescence is green. Thus, the ratio of red/green fluorescence is indicative of the mitochondrial membrane potential. Hippocampal slices were exposed to either $MPP^+$, 3-NP, NMDA, or rotenone. In these experiments, we used relatively high doses of each toxin. These toxin doses had been previously shown to produce significant cell death (with the exception of 3-NP) in organotypic slice cultures (see above). Summaries of the data are shown in Figures 1 and 2. Figure 1 shows the 1 hr time course of changes in the JC-1 fluorescence ratio following toxin exposure. Figure 2 shows a comparison of the groups 60 min after exposure. Only the Complex I inhibitors $MPP^+$ and rotenone caused significant mitochondrial depolarization 60 min after exposure. NMDA, which has been reported to induce MPT in neuronal cell cultures, had no effect of mitochondrial membrane potential in hippocampal slices. It should also be noted that the changes in mitochondrial membrane potential were much smaller than FCCP, a toxin that completely collapses the mitochondrial membrane potential.

At the doses used, all of the toxins produced complete and rapid inhibition of synaptic transmission in hippocampal slices, and except for 3-np, produce significant cell death in slice cultures. Thus, the data do not support the hypothesis that MPT occurs following toxin exposure. However, it remains possible that the small mitochondrial depolarization observed after exposure to Complex I inhibitors might reflect MPT in a small population of cells, since the method averages changes from a relatively large volume of tissue. It will be necessary to examine changes in mitochondrial membrane potential in single cells following toxin exposure. In future studies, we will use confocal microscopy to detect changes in mitochondrial membrane potential in single cells in organotypic slice cultures, where cell death can also be examined.

Other Indicators of MPT in Brain Slices – Because the mitochondrial toxins did not provide clear evidence of MPT in brain slices, we examined changes in mitochondrial membrane potential under conditions that have been shown to induce MPT in neuronal cell culture preparations. We examined four conditions: exposure to MPT inducing agents tert-butyl-hydroperoxide (t-BuOOH), thapsigargin (TG), zinc – high potassium, and oxygen-glucose deprivation (OGD). TG and t-BuOOH, at doses shown to cause MPT in neuronal cultures, had no effect on mitochondrial membrane potential in hippocampal slices. Zn-$K^+$, and to a greater extent OGD, caused mitochondrial depolarization in hippocampal slices. However, the change in mitochondrial membrane potential was not inhibited by Cyclosporin A, a signature inhibitor of MPT in a variety of cell types and in isolated mitochondria. These data are shown in Figures 3 and 4. We must tentatively conclude that MPT does not readily occur in hippocampal brain slices. It is possible that cells in intact brain acquired from adult animals are relatively resistant to MPT in comparison to isolated mitochondria and cultured cells acquired from relatively young animals.

Mitochondrial Reactive Oxygen Species (ROS) Production and Toxin Exposure

Mitochondria are thought to be an important source of free radical production in a variety of neurological conditions. To examine ROS production after exposure to $MPP^+$, rotenone, 3-NP, or NMDA, we used the fluorescent indicator dihydrorhodamine 123. Non-fluorescent dihydrorhodamine 123 is oxidized to fluorescent rhodamine 123 by $H_2O_2$. Hippocampal slices were loaded with dihydrorhodamine 123 and exposed to doses of $MPP^+$, rotenone, 3-NP, or NMDA that previously caused cell death in organotypic slice cultures. A summary of the results is shown in Figure 5. All toxins caused an increase in dihydrorhodamine 123 fluorescence. Statistical comparisons are provided in Figure 6 for data taken 60 min after toxin exposure. By 60 min, all toxins showed a significant increase in dihydrorhodamine 123 fluorescence compared to control.

These data indicate that ROS rproduction increases after exposure to Complex I inhibition, Complex II inhibition, or excitotoxicity. The data suggest that conditions would be favorable for MPT since mitochondrial oxidation promotes MPT in a variety of cell types and in isolated mitochondria. However, we found no indication of MPT in hippocampal slices, as described above.

**Key Research Accomplishments**

1) The Complex I inhibitors, $MPP^+$ and rotenone cause selective damage to pyramidal cells in hippocampal subfield CA1. This pattern of cell damage is similar to that produced by hypoxia/ischemia.
2) Only Complex I inhibition caused significant depolarization of mitochondrial membrane potential in hippocampal slices.
3) We have been unable to produce evidence of mitochondrial permeability transition in hippocampal slices.
4) All toxins tested produced increased dihydrorhodamine 123 fluorescence indicating increased ROS production.

## Reportable Outcomes

Xu, G.-P, Perez-Pinzon, M.A., and Sick, T.J. Mitochondrial Complex I Inhibition Produces Selective Damage to Hippocampal Subfield CA1, Neurotoxicity Research, Submitted for Publication.

## Conclusions

The results documented here indicate that inhibitors of mitochondrial Complex I kill pyramidal cells in the hippocampus and that damage is selective for the CA1 subfield. Complex I inhibitors also cause mitochondrial depolarization and production of reactive oxygen species in subfield CA1. However, although mitochondrial depolarization and ROS production might signal mitochondrial permeability transition (MPT), we have, as yet, no direct evidence that MPT occurs in hippocampus. This is an important point because MPT has been a major focus of research in the field of neurodegeneration.

## Appendix

Xu, G.-P., Perez-Pinzon, M.A., and Sick, T.J. Mitochondrial Complex I Inhibition Produces Selective Damage to Hippocampal Subfield CA1, Neurotoxicity Research, Submitted for Publication.

## Figure Legends

Figure 1. Effects of toxin exposure on mitochondrial membrane potential (JC-1 fluorescence) in hippocampal subfield CA1. Slices were incubated in JC-1 and then exposed to ACSF (Control), 1 mM $MPP^+$, 1 mM 3-NP, 100 uM NMDA, or 10 uM rotenone. Values are means ± SEM, n = 6.

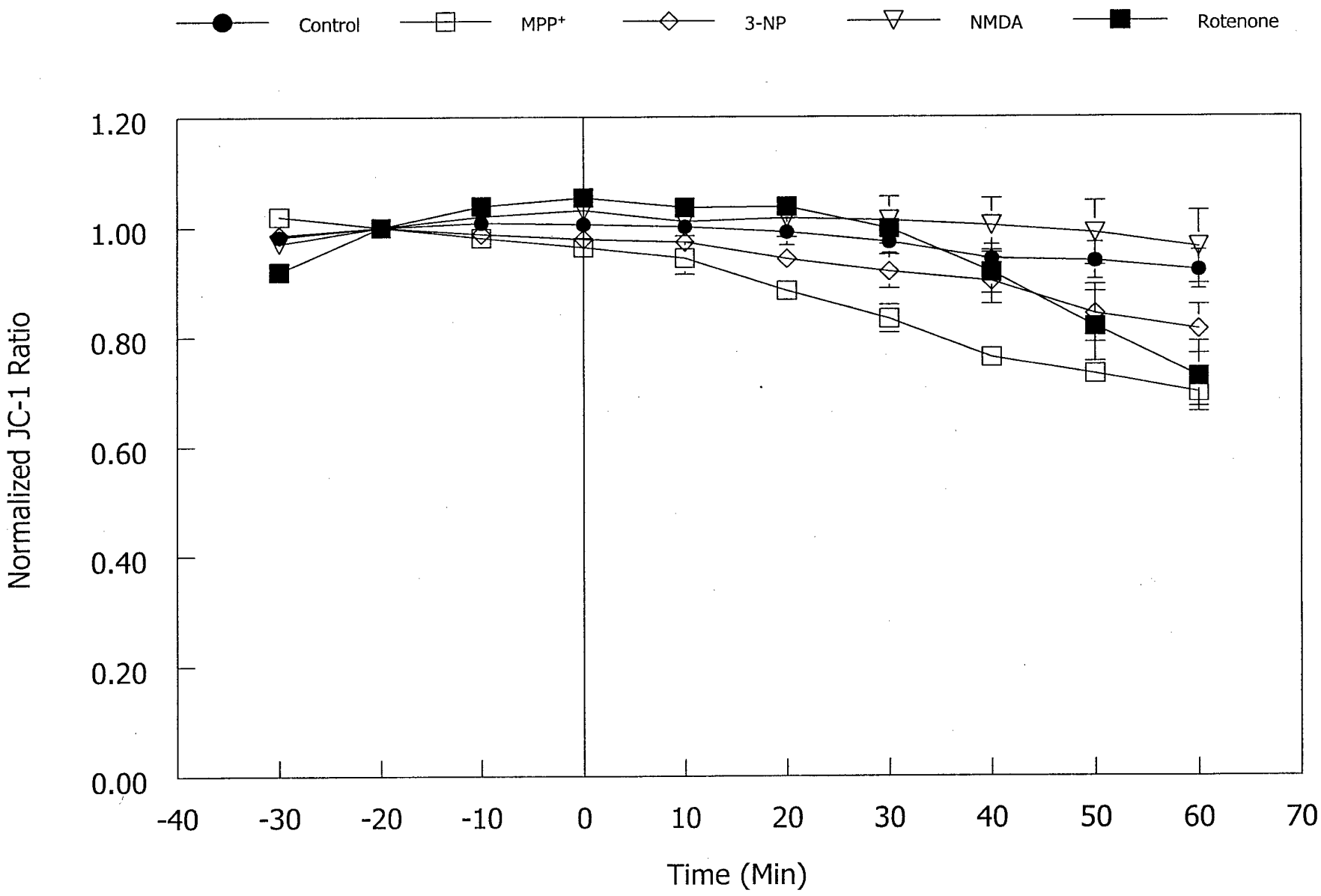
Figure 2. Comparison of relative mitochondrial membrane potential values (normalized JC-1 fluorescence ratios) 60 min after exposure to toxins (doses same as in Fig. 1). ** $p < .01$ compared with control.

Figure 3. Examples of the changes in mitochondrial membrane potential after exposure of hippocampal slices to 10 uM thapsigargin or 150 uM tert-butylhydroperoxide (t-BuOOH). These agents have been shown to promote mitochondrial permeability transition (MPT) in isolated mitochondria and cultured neurons.

Figure 4. Relative changes in mitochondrial membrane potential following exposure of hippocampal slices to 300 nM $Zn^+$ - 60 mM $K^+$ (top graph) or oxygen-glucose deprivation (lower graph) sufficient to cause 20 min of cellular depolarization (AD-anoxic depolarization). While both Zn-K and oxygen-glucose deprivation caused mitochondrial depolarization, the depolarization was not inhibited by Cyclosporin A (CsA).

Figure 5. Time course of changes in dihydrorhodamine 123 fluorescence following exposure of hippocampal slices to the indicated toxins. Toxin doses were the same as those shown in Figure 1.

Figure 6. Comparison of normalized dihydrorhodamine 123 fluroescence changes 60 min after toxin exposure. An increase in dihydrorhodamine fluorescence indicates increased production of reactive oxygen species. ** $p < .01$, * $p < .05$, $n = 5$.


Control
MPP+
3-NP
NMDA
Rotenone
Normalized JC-1 Ratio
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1.00
0.80
0.60
0.40
0.20
0.00
-40
-30
-20
-10
0
10
20
30
40
50
60
70
Time (Min)

Figure 1

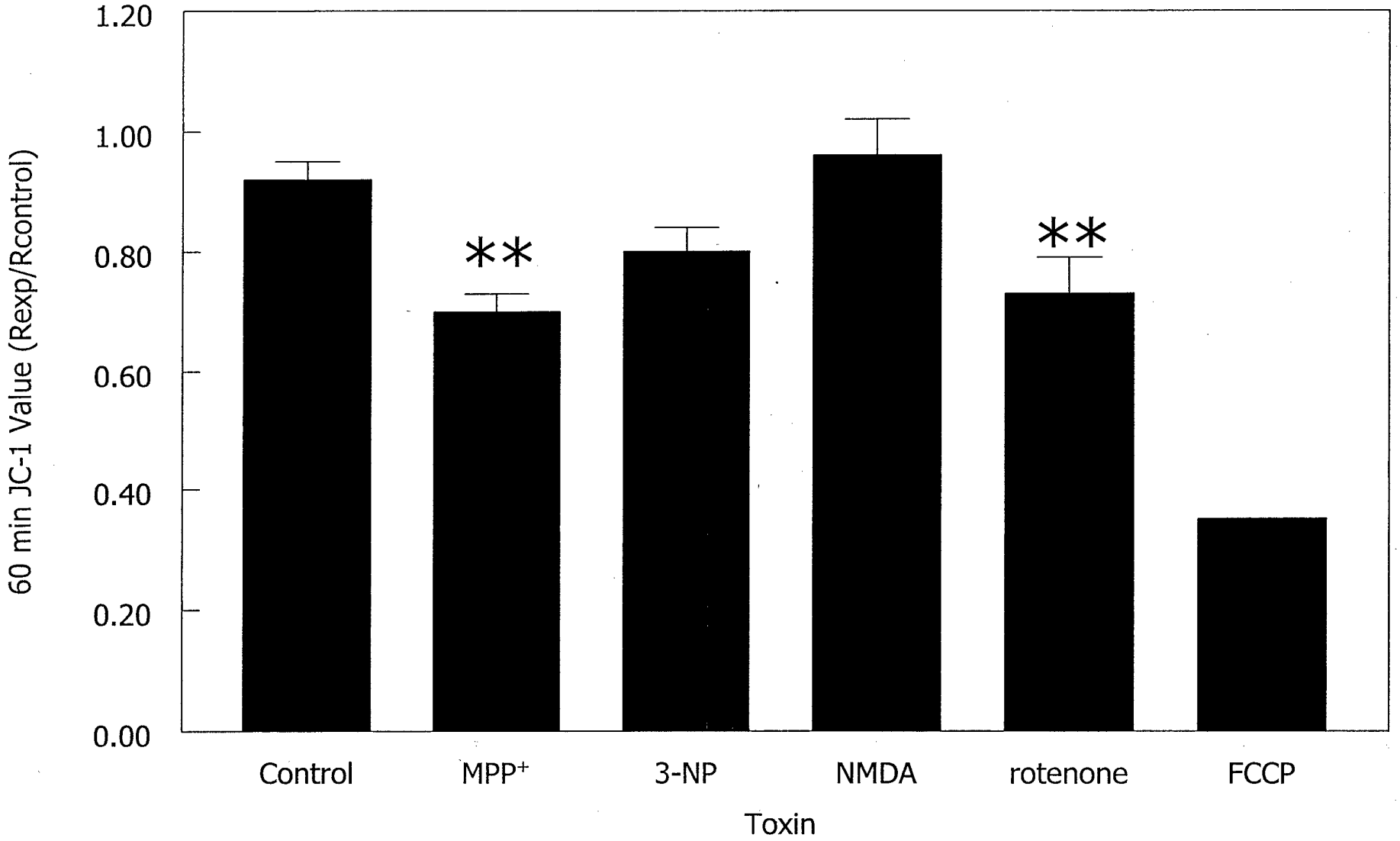

1.20
1.00
0.80
0.60
0.40
0.20
0.00
60 min JC-1 Value (Rexp/Rcontrol)
**
**
Control
MPP+
3-NP
NMDA
rotenone
FCCP
Toxin

Figure 2

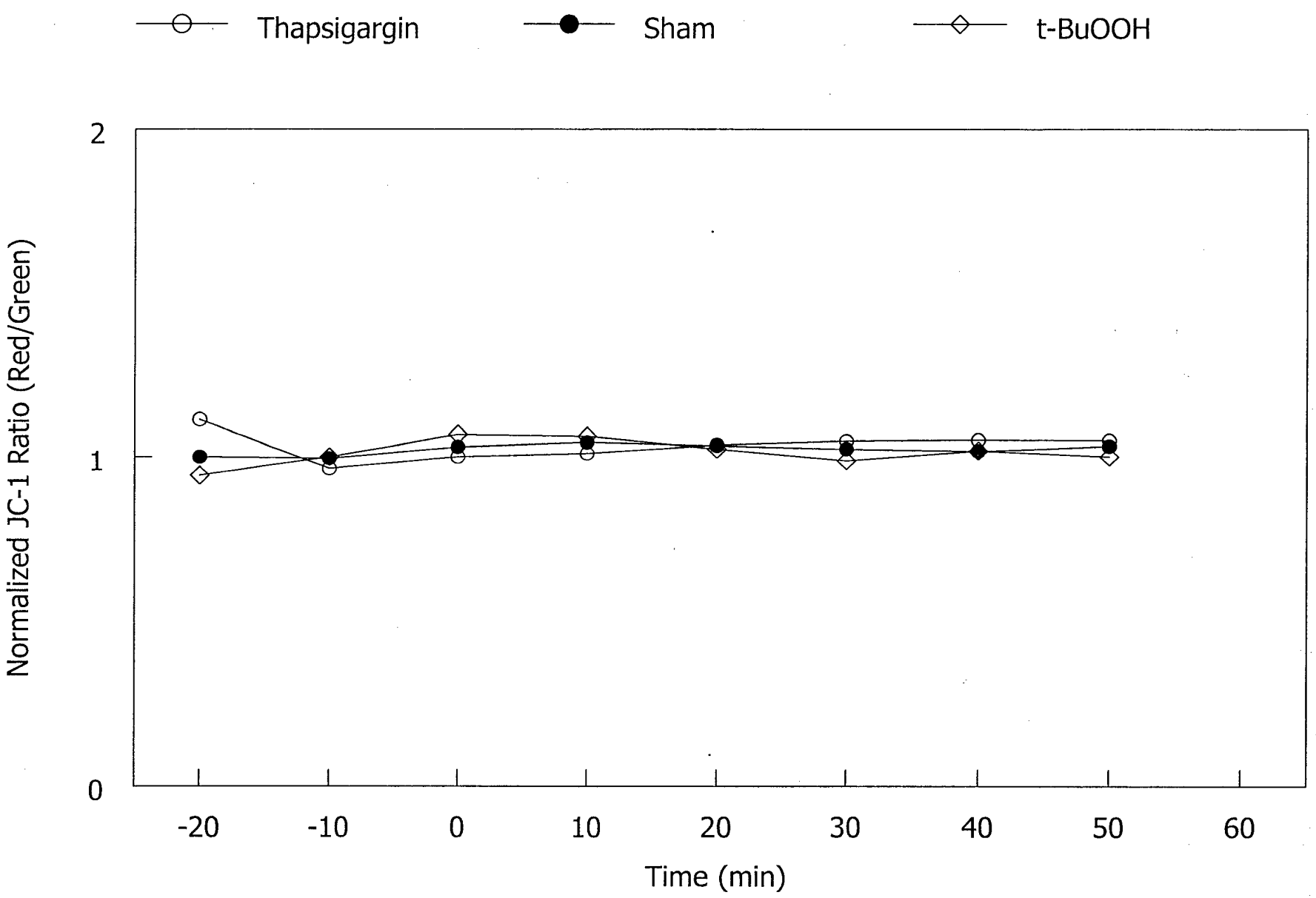
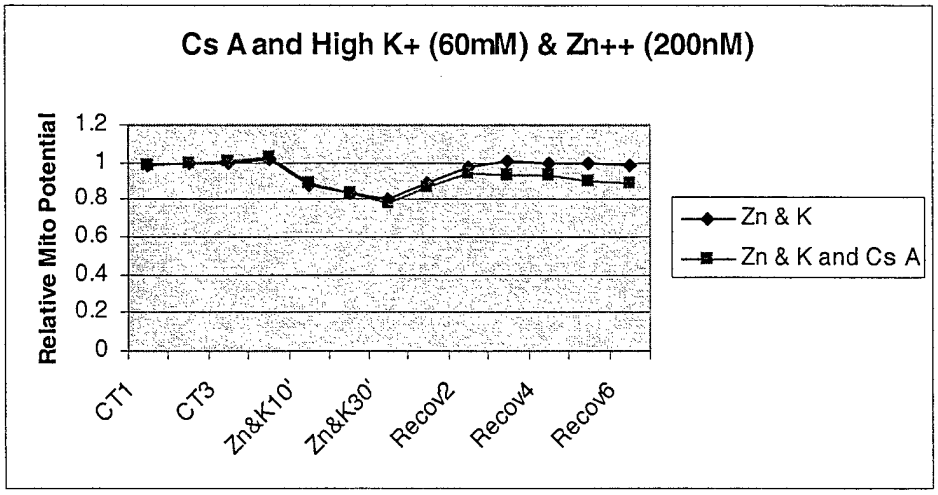

Thapsigargin
Sham
t-BuOOH
Normalized JC-1 Ratio (Red/Green)
2
1
0
-20
-10
0
10
20
30
40
50
60
Time (min)

Figure 3


Cs A and High K+ (60mM) & Zn++ (200nM)
Relative Mito Potential
1.2
1
0.8
0.6
0.4
0.2
0
CT1
CT3
Zn&K10'
Zn&K30'
Recov2
Recov4
Recov6
Zn & K
Zn & K and Cs A

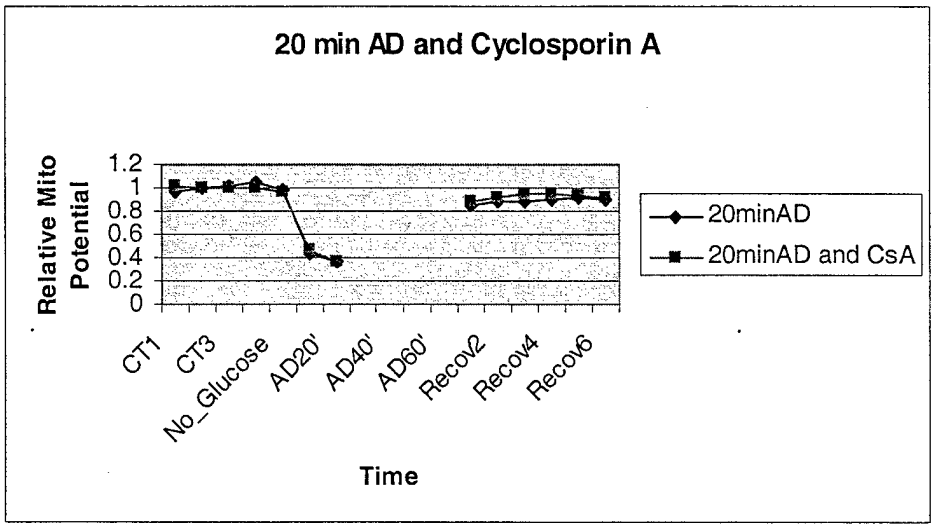

20 min AD and Cyclosporin A
Relative Mito Potential
1.2
1
0.8
0.6
0.4
0.2
0
CT1
CT3
No_Glucose
AD20'
AD40'
AD60'
Recov2
Recov4
Recov6
Time
20minAD
20minAD and CsA

Figure 4

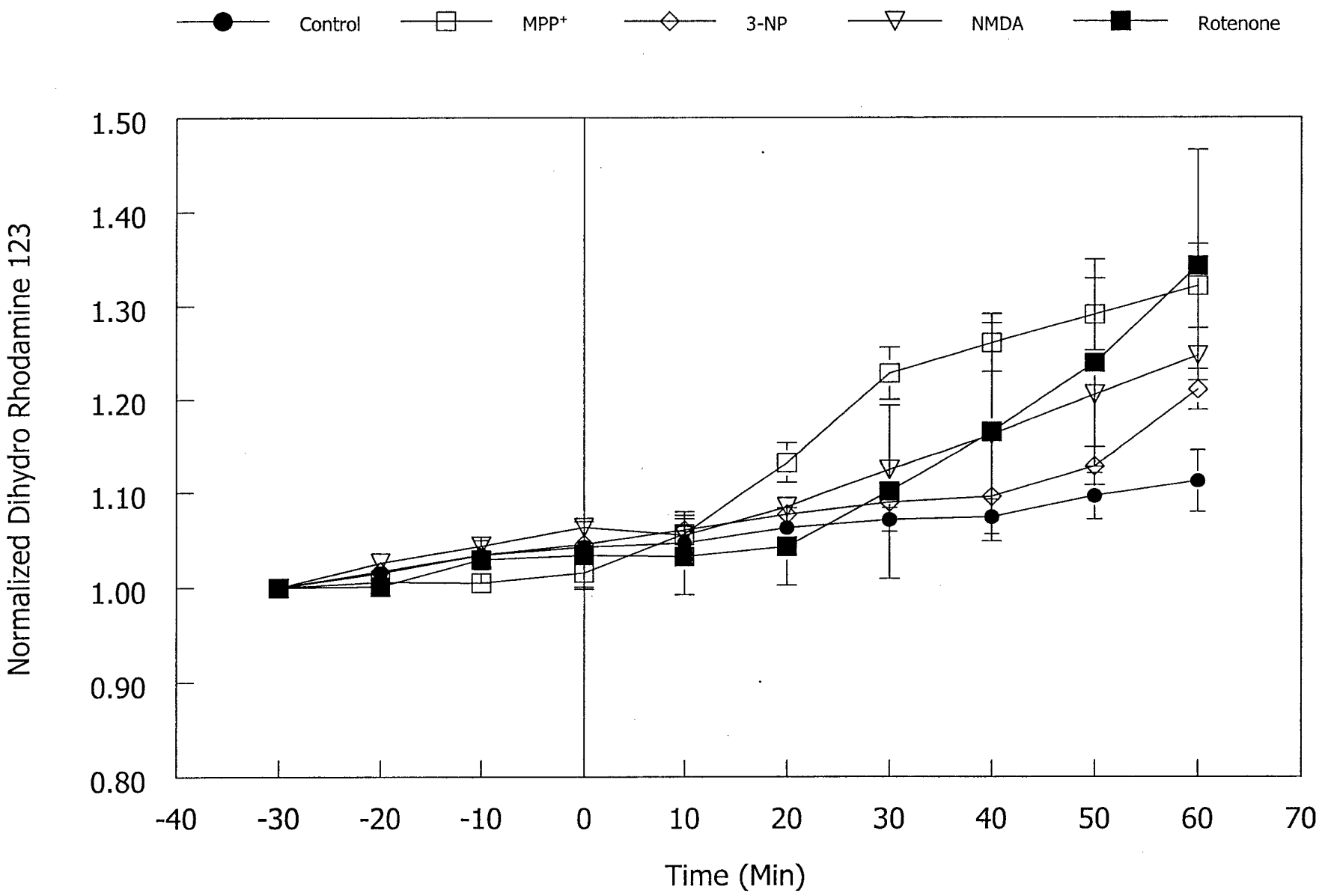

Control
MPP+
3-NP
NMDA
Rotenone
Normalized Dihydro Rhodamine 123
1.50
1.40
1.30
1.20
1.10
1.00
0.90
0.80
-40
-30
-20
-10
0
10
20
30
40
50
60
70
Time (Min)

Figure 5

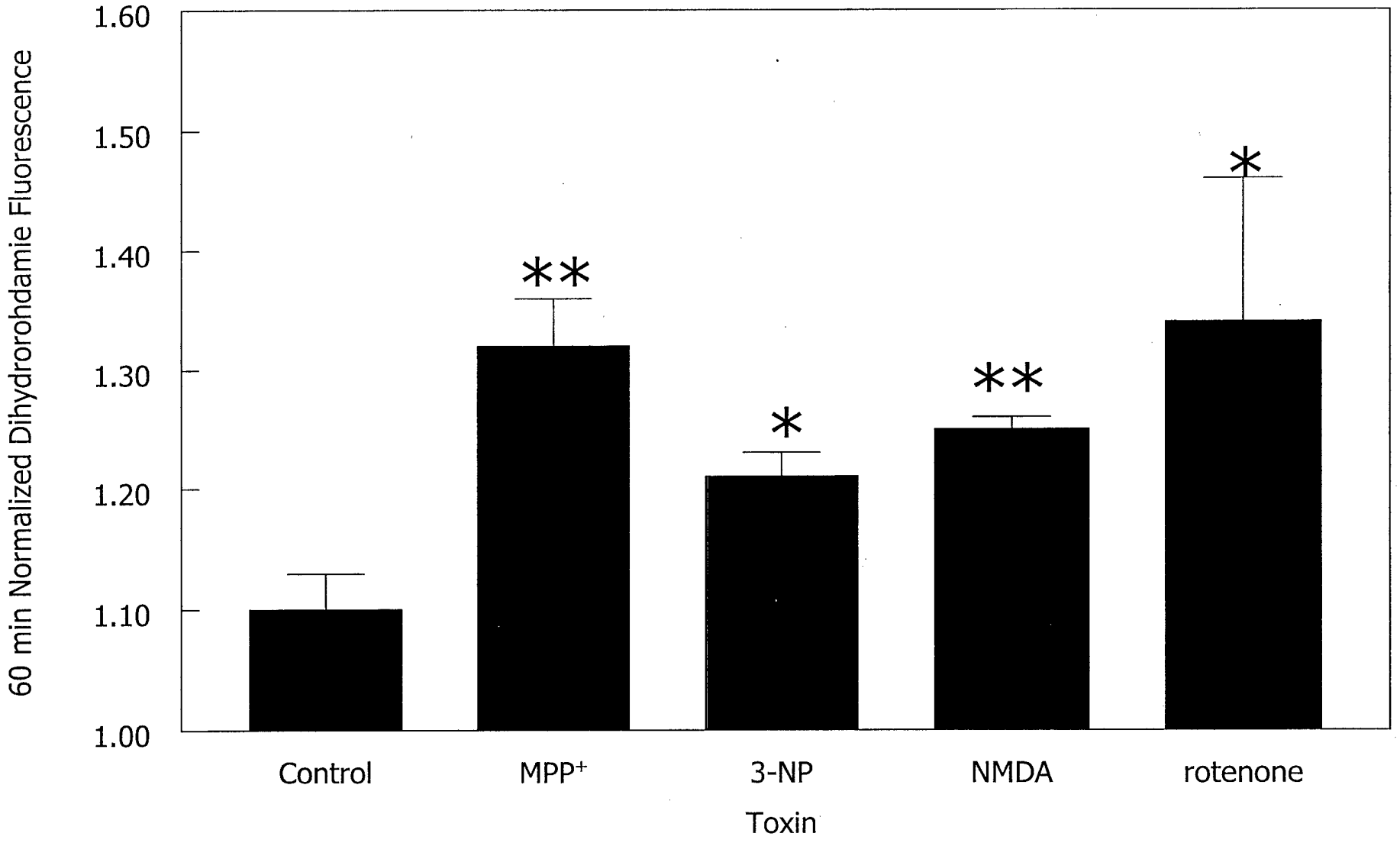

60 min Normalized Dihydrorohdamie Fluorescence
1.60
1.50
1.40
1.30
1.20
1.10
1.00
**
*
**
*
Control
MPP+
3-NP
NMDA
rotenone
Toxin

Figure 6

# Mitochondrial Complex I Inhibition Produces Selective Damage to Hippocampal Subfield CA1 in Organotypic Slice Cultures

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## ABSTRACT

The effects of mitochondrial respiratory chain inhibitors and the excitotoxin N-methyl-D-aspartate (NMDA) on cell death in hippocampal subfields CA1 and CA3 were examined in hippocampal organotypic slice cultures. 2-3 week old slice cultures were exposed for one hour to either the Complex I inhibitors, rotenone or 1-methyl-4-phenylpyridium ($MPP^+$), the Complex II inhibitor 3-nitropropionic acid (3-NP), or the excitotoxin NMDA. Cell death was examined 1, 2, or 3 days following treatment by measuring propidium iodide (PI) fluorescence. Rotenone, and to a lesser extent $MPP^+$ caused greater cell death in hippocampal subfield CA1 than CA3. NMDA caused similar damage in both hippocampal subfields while 3-NP produced little damage in either subfield. The data suggest that mitochondrial Complex I inhibition can produce selective cell damage in hippocampus and in this regard is similar to that observed following hypoxia/ischemia.



Key Words: selective neuronal vulnerability, mitochondrial toxins, excitotoxicity.

# INTRODUCTION

Many neurological disorders are characterized by degeneration of specific neuronal populations. Global ischemia results in selective damage to hippocampal pyramidal cells in the CA1 subfield, dorsolateral striatum, and cortex (Kirino, 1982; Pulsinelli, 1982; Kirino, 2000). Parkinson's disease is characterized by selective damage to dopaminergic neurons in the substantia nigra (Betarbet, 2002). Similarly, selective neuronal degeneration of neurons in nucleus basalis of Maynert and cortical motor neurons are common features of Alzheimer's disease (Whitehouse et al., 1981) and amyotropic lateral sclerosis (Eisen and Weber, 2001) respectively.

Mitochondria have been implicated as mediators of neuronal death in a variety of neurological conditions (Greenamyre et al., 2001; Beal, 2001; Fiskum, 2000). Patients with Parkinson's disease showed deficiencies of respiratory chain Complex I (Schapira, 1989) and systemic administration of Complex I inhibitors or their precursors produced degeneration of dopaminergic neurons in the substantia nigra in animals (Betarbet, 2002) and man (Langston et al., 1999). Selective damage occurs to dopaminergic neurons in the substantia nigra following systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) occurs because MPTP is oxidized to the toxic metabolite $MPP^+$ (Langston et al., 1984) which is transported into dopaminergic neurons (Javitch et al., 1985). Toxicity of $MPP^+$ appears to be, at least in part, due to its abilitiy to inhibit Coomplex I (Nicklas et al., 1987). Another Complex I inhibitor, rotenone, selectively damages dopaminergic neurons in the substantia nigra although it apparently inhibits Complex I uniformly throughout the brain (Betarbet et al., 2000).

In the present study, we examined the hypothesis that Complex I inhibition would selectively damage non-dopaminergic neurons previously shown to be vulnerable to mitochondrial inhibition from hypoxia/ischemia. We demonstrate here that exposure of hippocampal organotypic slice cultures to Complex I inhibitors results in selective damage to CA1 pyramidal cells similar to that reported following hypoxia/ischemia.

## MATERIALS AND METHODS

### Preparation of Cultures

Organotypic slice cultures of the hippocampus were prepared according to the methods described by Bergold and Casaccia-Bonnefil (1997) and the protocol was approved by the University of Miami, Animal Care and Use Committee. Neonatal Sprague Dawley rats (9-11 days old) were anesthetized by single intraperitoneal injections of ketamine (1.0 mg/pup). The pups were decapitated and the hippocampi dissected free from the cerebral hemispheres and transversely sliced (400 $\mu$m) on a McIlwain tissue chopper. Slices were incubated in Gey's Balanced Salt Solution (Gibco/Life Technologies) supplemented with 6.5 mg/ml glucose (Sigma Chemical) for 1 hour at 4$^{o}$ C. Slices then were then transferred onto 30-mm diameter membrane inserts (Millicell-CM, Millipore), and were transferred to 6 well culture trays with 1 ml of slice culture medium per well. The slice culture medium consisted of 50 % Minimum Essential Medium (Gibco/Life Technologies), 25 % Hank's balanced salt solution

(Gibco/Life Technologies), 25 % heat inactivated horse serum (Gibco/Life Technologies) supplemented with 6.5 mg/ml glucose and glutamine. The cultures were maintained at 37 °C in an incubator (Nuaire, CF autoflow) with an atmosphere of humidified 21% $O_2$ - 5% $CO_2$. The slice culture medium was changed twice a week and slices were cultured for 14-15 days before experiments.

**Assessment of Cell Death**

The number of dead or dying cells in hippocampal subfields CA1 and CA3 was estimated by fluorescent staining with Propidium Iodide (PI, from Sigma). Prior to experimental treatment (exposure to mitochondrial inhibitors), slices were incubated in culture medium supplemented with 2 $\mu$g/ml PI for 1 hr. Images of PI fluroescence in organotypic slices were acquired using an inverted fluorescence microscope (Olympus IX 50) and Spot CCD camera (Diagnostic Instruments Inc., Sterling Heights MI) and SPOT advanced software. Images of cultured slices were taken prior to toxin exposure, to estimate background fluorescence, and again 24 hr, 48 hr, and 72 hr later. Finally, the slices were superfused with NMDA (100 μM)(from Sigma) for 1 hr and a terminal image was acquired 24 hr later to estimate maximum cell death. For quantitation, regions of interest (ROIs) corresponding to hippocampal subfields CA1 and CA3 were selected from bright field images of each slice using Scion Image software (Windows Version). ROIs were transferred to individual images in an image stack after optical allignment of brightfield images. Relative cell death was calculated from each ROI as follows: Relative % cell death = (Fexp - Fmin)/(Fmax - Fmin)* 100, where Fexp is the fluorescence of the test condition, Fmax is maximum fluorescence observed during an

experiment (following toxin exposure or terminal treatment with 100 μM NMDA for 1 hr), and Fmin is background fluorescence (prior to treatment).

**Mitochondrial Inhibitors**

Hippocampal organotypic slice cultures were treated for 1 hr with either the Complex I inhibitors rotenone (0.1 μM, 1 μM ,10 μM ) or 1-methy-4-phenylpyridinium ($MPP^+$, 10 μM, 100μM, 1 mM ), the Complex II inhibitor 3-nitropropionic acid (10 μM, 100μM, 1 mM ), or the excitotoxin NMDA (1 μM ,10 μM, 100μM). All compounds were dissolved in culture medium (see above) except rotenone which was dissolved in culture medium containing 0.1% ethanol. Sham treated slices were incubated for 1 hr in exchanged culture medium or culture medium containing 0.1% ethanol (vehicle for rotenone).

**Statistics**

Experimental groups were compared statistically using Analysis of Variance (ANOVA).

## RESULTS

Hippocampal organotypic slice cultures were a reliable model for assessing cell death following exposure to mitochondrial toxins. Little background PI fluorescence was observed in hippocampal subfields CA1 And CA3 prior to toxin exposure or in sham-treated slices, indicating limited cell death in untreated slices after 2-3 weeks of culture. However, many slices showed some damaged cells in the upper blade of the dentate

gyrus prior to toxin exposure. The variability in the dentate gyrus precluded any meaningful assessment of the effects of toxin exposure in this hippocampal subfield.

**Rotenone**

Hippocampal slices were exposed for 1 hr either to vehicle, 0.1 μM, 1.0 μM, or 10 μM concentrations of the mitochondrial Complex I inhibitor, rotenone. Examples of images showing the changes in PI fluroescence taken prior to exposure, and each of 3 days after exposure, are shown in figure 1. Quantitative assessment of cell death in hippocampal subfields CA1 and CA3 following rotenone exposure are shown in Figure 2. Rotenone treatment resulted in a highly significant dose-dependent increase in cell death in hippocampal pyramidal cells both in CA1 and CA3 (Main effect of dose, $F = 84.5$, df 3,24, $p < .001$, n= 4). Significantly, cell death following rotenone exposure was more pronounced at lower doses in subfield CA1 than in subfield CA3 (Region X Dose interaction, $F = 5.4$, df 3,24, $p < .01$, $n = 4$). For example, PI fluorescence following exposure to 1 μM rotenone was 30.1 ± 11.1 %, 77.1 ± 10.9 % and 85.1 ±16.5 % on days 1, 2, an 3 respectively in CA1, and 9.4 ± 5.5 %, 20.3 ± 10.7, and 58.5 ± 20.5% on days 1, 2, an 3 in CA3. Higher doses of rotenone (10 μM) resulted in significant cell death in both hippocampal subfields.

**1-Methyl-4-Phenylpyridium ($MPP^+$)**

Examples of PI fluorescence images of slice cultures exposed to 10 μM, 100 μM, or 1 mM of the respiratory chain Complex I inhibitor $MPP^+$, are shown in figure 3. Quantitative analysis of PI fluorescence following $MPP^+$ exposure is shown in figure 4. Similar to rotenone, $MPP^+$ caused dose-dependent death of pyramidal cells in both CA1 and CA3 (Main Effect of Dose, $F = 10.2$, df 3,24, $p < 0.001$, $n = 4$). After exposure to 1

mM $MPP^+$, cell death as indicated by PI fluorescence was 42.8 ± 17.1 %, 75.5 ± 23.8 %, and 73.3 ± 20.6 % on days 1, 2, and 3 respectively in subfield CA1. In subfield CA3, PI fluroescence was 25.0 ± 14.5 %, 44.7 ± 26.4 %, and 42.3 ± 17.8 % on days 1, 2, and 3 after exposure to 1 mM $MPP^+$. However, while there was a trend for damage to be greater in CA1 than CA3 following $MPP^+$ exposure, the difference did not reach statistical significance (Dose X Region interaction , $F = 1.3$, df 3,24, $p = 0.3$, $n = 4$).

**3-Nitropropionic Acid (3-NP)**

In comparison to the Complex I inhibitors rotenone and $MPP^+$, the Complex II inhibitor 3-NP had a much smaller effect on pyramidal cell death (Main Effect of Dose, $F = 3.1$, df 3,24, $p < .05$, $n = 4$). Also, there was no tendency for 3-NP to selectively damage either subfield (Dose X Region interaction, $F = 0.6$, df 3,24, $p = 0.6$, $n = 4$). A summary of the data is presented in figure 5 (Images not shown).

**N-Methyl-D-Aspartate (NMDA)**

The excitotoxin NMDA caused pyramidal cell death only at the highest dose (100 μM) tested (Main Effect of Dose, F 416, df 3,24, $p < .0001$, $n = 4$). Examples of PI fluorescence images acquired before and after exposure of organotypic slice cultures to 1 μM , 20 μM , or 100 μM NMDA are shown in figure 6. A summary of the data is shown in figure 7. In contrast to rotenone, and tentatively $MPP^+$, NMDA did not produce selective damage in subfield CA1 compared to CA3 (Dose X Region interaction $F = 1.5$, df 3,24, $p = 0.2$, $n = 4$).

## DISCUSSION

In this report we demonstrate that inhibition of mitochondrial respiratory chain Complex I results in selective damage to pyramidal cells in subfield CA1. Selective neuronal damage was not observed following inhibition of Complex II with 3-NP or following exposure to the excitotoxin NMDA. We believe this is the first report that Complex I inhibition causes selective damage to CA1 pyramidal cells in the hippocampus.

Alterations in Complex I activity of the mitochondrial respiratory chain have typically been associated with Parkinson's disease and damage to dopaminergic neurons in the substantia nigra and striatum. MPTP caused selective damage to neurons in the substantia nigra and striatum in man (Langston et al., 1999), animals (Betarbet, 2002) and produced neurological symptoms similar to those found in Parkinson's disease (Langston et al., 1983). MPTP appears to selectively damage dopaminergic neurons

though its conversion to the Complex I inhibitor $MPP^+$ (Langston et al., 1984), and transport of $MPP^+$ selectively into synaptic terminals of dopaminergic neurons (Javitch et al., 1985). It has also been reported that patients with Parkinson's disease may show a deficiency in mitochondrial Complex I ((Schapira, 1989; Parker et al., 1989). More recently, it was reported that chronic systemic administration of rotenone, another Complex I inhibitor, caused selective degeneration of dopaminergic neurons in the striatum and substantia nigra (Betarbet et al., 2000).

In contrast, selective damage to pyramidal cells in hippocampal subfield CA1 has been commonly reported following global ischemia in vivo (Kirino, 1982, and Kirino, 2000). Oxygen-glucose deprivation (in vitro ischemia) has also been shown to selectively damage CA1 pyramidal cells in hippocampal organotypic slice cultures (Laake et al., 1999). We report here that selective damage to CA1 pyramidal cells also occured after treatment of hippocampal organotypic slice cultures with the Complex I inhibitor rotenone. Betarbet at al (2000) did not report damage to CA1 pyramidal cells after systemic rotenone infusion *in vivo*. However it was unclear whether damage did not occur or whether a detailed analysis of hippocampus pathology was not conducted. Also, in their study exposure to rotenone was chronic and at a lower dose than used here in organotypic slice cultures.

The mechanism of rotenone-induced cell damage in hippocampal subfield CA1 is currently unknown. However, because a similar pattern of damage occurs with oxygen-glucose deprivation and rotenone treatment, it is safe to speculate that the mechanism of damage also is similar. One such possibility is the generation of reactive oxygen species (ROS). Oxygen-glucose deprivation results in increase ROS generation in organotypic

hippocampal slice cultures (Frantseva et al., 2001). Complex I inhibition with rotenone also increase ROS production in hippocampal slices (Saybasili et al., 2001). However, if ROS production is key to selective neuronal damage in CA1 pyramidal cells, the increase should be greater in CA1 than in CA3 following either oxygen-glucose deprivation or Complex I inhibition. To date, selective ROS production in CA1 has not been examined.

Elevation of intracellular calcium could also mediate selective neuronal vulnerability of CA1 pyramidal cells following either Complex I inhibition or oxygen-glucose deprivation. Intracellular calcium levels were clearly elevated following oxygen glucose deprivation (Frantseva et al. 2001) and more importantly the increase occurred earliest and was most severe in subfield CA1 (Mitani, 1994). Complex I inhibition also results in elevation of intracellular calcium (Leist et al., 1998) but experiments have not been conducted to compare changes in hippocampal subfields CA1 and CA3.

We did not observe reliable selective hippocampal CA1 pyramidal cell death following treatment either with the excitotoxin, NMDA, or the mitochondrial Complex II inhibitor 3-NP. Previous studies (Kristensen et al., 2001; Prendergast et al., 2001) also showed comparable cell death in subfields CA1 and CA3 following exposure to high (>100 μM) concentrations of NMDA. However, Kristensen et al (2001) reported selective damage to CA1 pyramidal cells after 2 days of exposure to 10 μM NMDA. There have been no previous studies examining selective neuronal vulnerability in hippocampus following inhibition of mitochondrial Complex II. In fact, treatement with 3-NP has been shown to "pre-condition" or protect hippocampal neurons from subsequent hypoxia/ischemia (Sugino et al. 1999).

In conclusion, we have demonstrated selective damage to pyramidal cells in subfield CA1 of hippocampal organotypic slice cultures following inhibition of Complex I of the mitochondrial respiratory chain. Selective damage to neurons in subfield CA1 was similar to that observed by other investigators after hypoxia/ischemia. Thus, it appears that severe inhibition of respiratory chain function may be sufficient to trigger CA1 neuronal death, whether inhibition occurs as a result of ischemia or exposure to mitochondrial toxins.

***acknowledgements***

This research was supported in part by grants from the Department of Defense (DAMD17-1-99-9493) and Public Health Service (NS38276).

## REFERENCES

Beal MF. (2001) Mitochondria and oxidative damage in amyotrophic lateral sclerosis. Funct. Neurol. **16,** Suppl. 4:161-169.

Bergold PJ, Casaccia-Bonnefil P. (1997) Preparation of organotypic hippocampal slice cultures using the membrane filter method. Methods Mol. Biol. **72,** 15-22.

Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. (2000) Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat. Neurosci. **3,** 1301-1306.

Betarbet R, Sherer TB, Greenamyre JT. (2002) Animal models of Parkinson's disease. Bioessays **24**, 308-318.

Eisen A, Weber M. (2001) The motor cortex and amyotrophic lateral sclerosis. Muscle Nerve **24,** 564-573.

Fiskum G, Murphy AN, Beal MF. (1999) Mitochondria in neurodegeneration: acute ischemia and chronic neurodegenerative diseases. J. Cereb. Blood Flow Metab.**19,** 351-369.

Frantseva MV, Carlen PL, Perez Velazquez JL. (2001) Dynamics of intracellular calcium and free radical production during ischemia in pyramidal neurons. Free Radic. Biol. Med. **15**, 1216-1227.

Greenamyre JT, Sherer TB, Betarbet R, Panov AV. (2001) Complex I and Parkinson's disease. IUBMB Life **52**, 135-411.

Javitch JA, Snyder SH. (1984) Uptake of MPP(+) by dopamine neurons explains selectivity of parkinsonism-inducing neurotoxin, MPTP. Eur. J. Pharmacol. **13**,455-456.

Kirino T. (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res. **239**, 57-69.

Kirino T. (2000) Delayed neuronal death. Neuropathology. **20 Suppl**, S95-S97.

Kristensen BW, Noraberg J, Zimmer J. (2001) Comparison of excitotoxic profiles of ATPA, AMPA, KA and NMDA in organotypic hippocampal slice cultures. Brain Res. **917**, 21-44.

Laake JH, Haug FM, Wieloch T, Ottersen OP. (1999) A simple in vitro model of ischemia based on hippocampal slice cultures and propidium iodide fluorescence. Brain Res Brain Res. Protoc. **4**, 173-184.

Langston JW, Irwin I, Langston EB, Forno LS. (1984) Pargyline prevents MPTP-induced parkinsonism in primates. Science. **225**, 1480-14822.

Langston JW, Ballard P, Tetrud JW, Irwin I. (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science. **219**, 979-980.

Langston JW, Forno LS, Tetrud J, Reeves AG, Kaplan JA, Karluk D. (1999) Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. Ann. Neurol. **46**, 598-605.

Leist M, Volbracht C, Fava E, Nicotera P. (1998) 1-Methyl-4-phenylpyridinium induces autocrine excitotoxicity, protease activation, and neuronal apoptosis. Mol. Pharmacol. **54**, 789-801.

Mitani A, Takeyasu S, Yanase H, Nakamura Y, Kataoka K. (1994) Changes in intracellular Ca2+ and energy levels during in vitro ischemia in the gerbil hippocampal slice. J. Neurochem. **62**, 626-634.

Nicklas WJ, Youngster SK, Kindt MV, Heikkila RE. (1987) MPTP, MPP+ and mitochondrial function. Life Sci. **23**, 721-729.

Parker WD Jr, Boyson SJ, Parks JK. (1989) Abnormalities of the electron transport chain in idiopathic Parkinson's disease. Ann. Neurol. **26**, 719-723.

Prendergast MA, Harris BR, Mayer S, Holley RC, Pauly JR, Littleton JM. (2001) Nicotine exposure reduces N-methyl-D-aspartate toxicity in the hippocampus: relation to distribution of the alpha7 nicotinic acetylcholine receptor subunit. Med. Sci. Monit. **7**, 1153-1160

Pulsinelli WA, Brierley JB, Plum F. (1982) Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann. Neurol. **11**, 491-498.

Saybasili H, Yuksel M, Haklar G, Yalcin AS. (2001) Effect of mitochondrial electron transport chain inhibitors on superoxide radical generation in rat hippocampal and striatal slices. Antioxid. Redox. Signal. **3**, 1099-1104.

Schapira AH, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD. (1989) Mitochondrial complex I deficiency in Parkinson's disease. Lancet.**1**, 1269.

Whitehouse PJ, Price DL, Clark AW, Coyle JT, DeLong MR. (1981) Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis. Ann. Neurol. **10**, 122-126.

## FIGURE LEGENDS

Figure 1. Examples of brightfield and propidium iodide (PI) fluroescence images of hippocampal organotypic slice cultures. Rotenone (10 μM, 1 μM, or 0.1 μM) was applied for 1 hr after acquiring background images on Day 0. Final PI fluorescence was obtained 24 hr after applying 100 μM NMDA for 1 hr on Day 3.

Figure 2. Average values (Mean ± SEM) of propidium iodide (PI) fluorescence in hippocampal subfields CA1 and CA3 on Days 1, 2, and 3 after exposure to either vehicle (0.1% ethanol), 0.1 μM, 1.0 μM, or 10 μM rotenone. Statistical comparisons of groups are provided in the text.

Figure 3. Examples of brightfield and propidium iodide (PI) fluroescence images of hippocampal organotypic slice cultures. 1-methy-4-phenylpyridium ($MPP^+$, 1 mM, 100 μM, or 10 μM) was applied for 1 hr after acquiring background images on Day 0. Final PI fluorescence was obtained 24 hr after applying 100 μM NMDA for 1 hr on Day 3.

Figure 4. Average values (Mean ± SEM) of propidium iodide (PI) fluorescence in hippocampal subfields CA1 and CA3 on Days 1, 2, and 3 after exposure to 1 mM, 100 μM, or 10 μM $MPP^+$. Statistical comparisons of groups are provided in the text.

Figure 5. Examples of brightfield and propidium iodide (PI) fluroescence images of hippocampal organotypic slice cultures. N-methyl-D-aspartate (NMDA, 100 μM, 10 μM or 1 μM) was applied for 1 hr after acquiring background images on Day 0. Final PI fluorescence was obtained 24 hr after applying 100 μM NMDA for 1 hr on Day 3.

Figure 6. Average values (Mean ± SEM) of propidium iodide (PI) fluorescence in hippocampal subfields CA1 and CA3 on Days 1, 2, and 3 after exposure to 100 μM, 10 μM, or 1 μM NMDA. Statistical comparisons of groups are provided in the text.

Figure 7. Average values (Mean ± SEM) of propidium iodide (PI) fluorescence in hippocampal subfields CA1 and CA3 on Days 1, 2, and 3 after exposure to 1 mM, 100 μM, or 10 μM, 3-nitropropionic acid (3-NP). Statistical comparisons of groups are provided in the text.

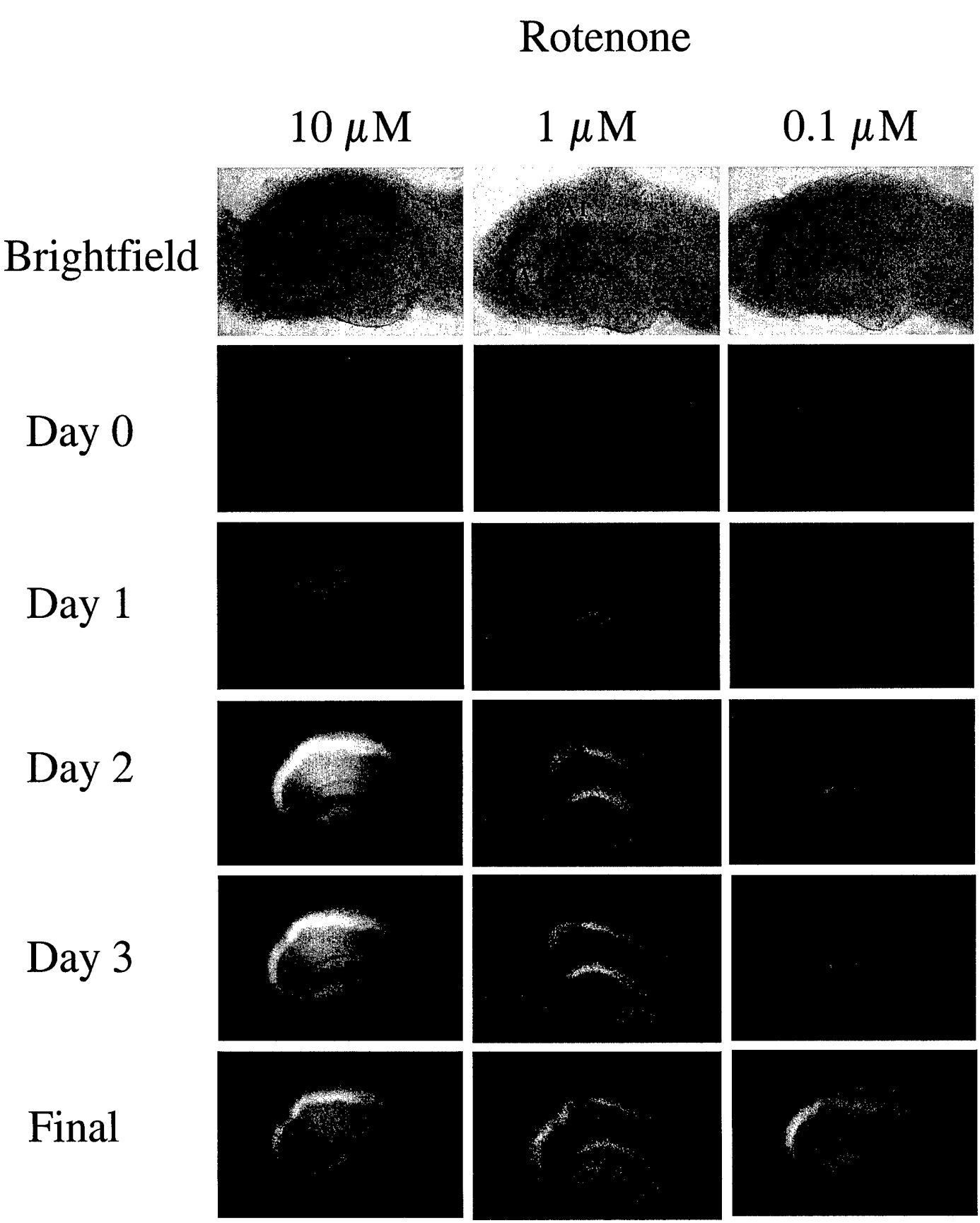
Rotenone
10 μM
1 μM
0.1 μM
Brightfield
Day 0
Day 1
Day 2
Day 3
Final

Figure 1

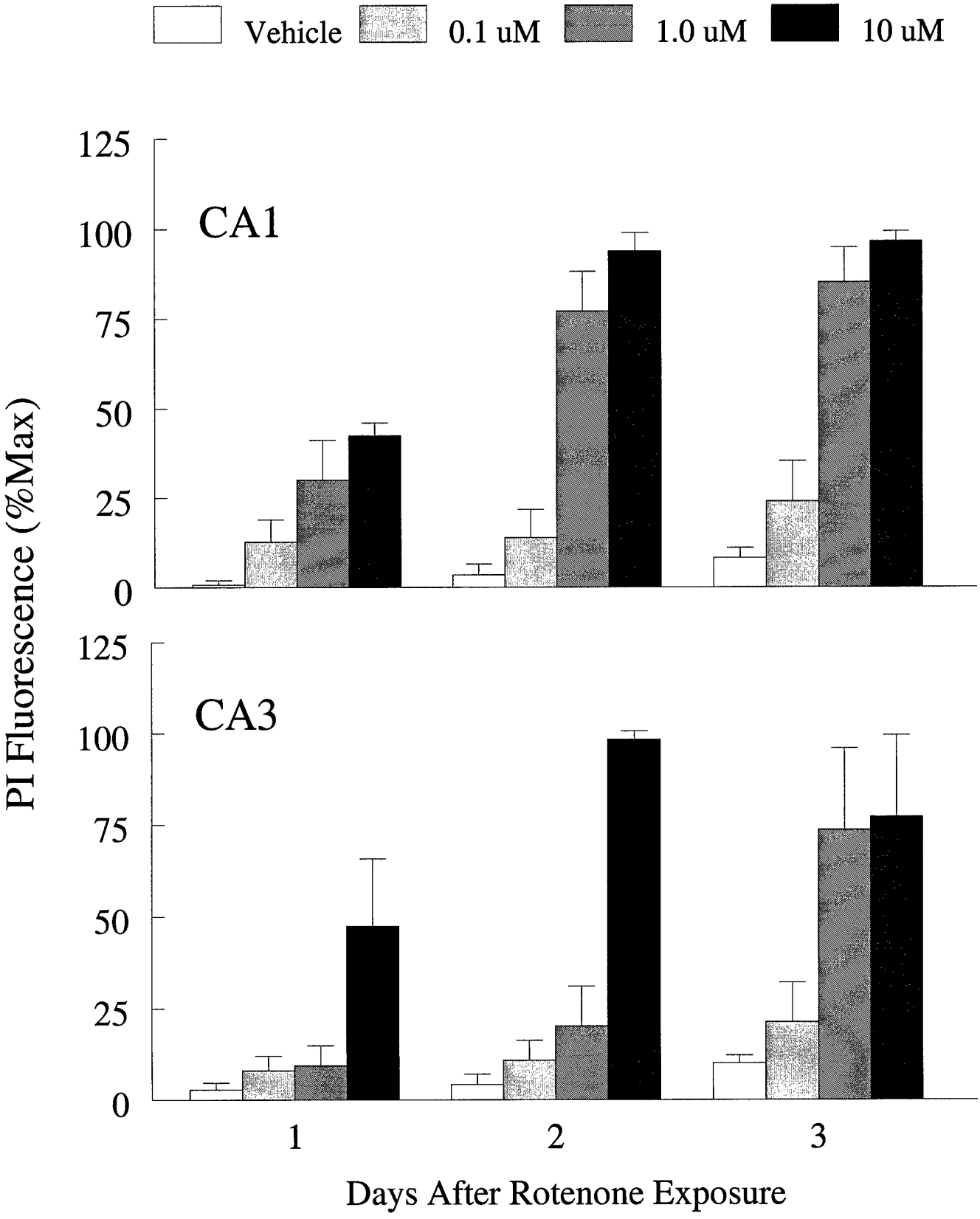
Vehicle
0.1 uM
1.0 uM
10 uM
CA1
CA3
PI Fluorescence (%Max)
125
100
75
50
25
0
1
2
3
Days After Rotenone Exposure

Figure 2

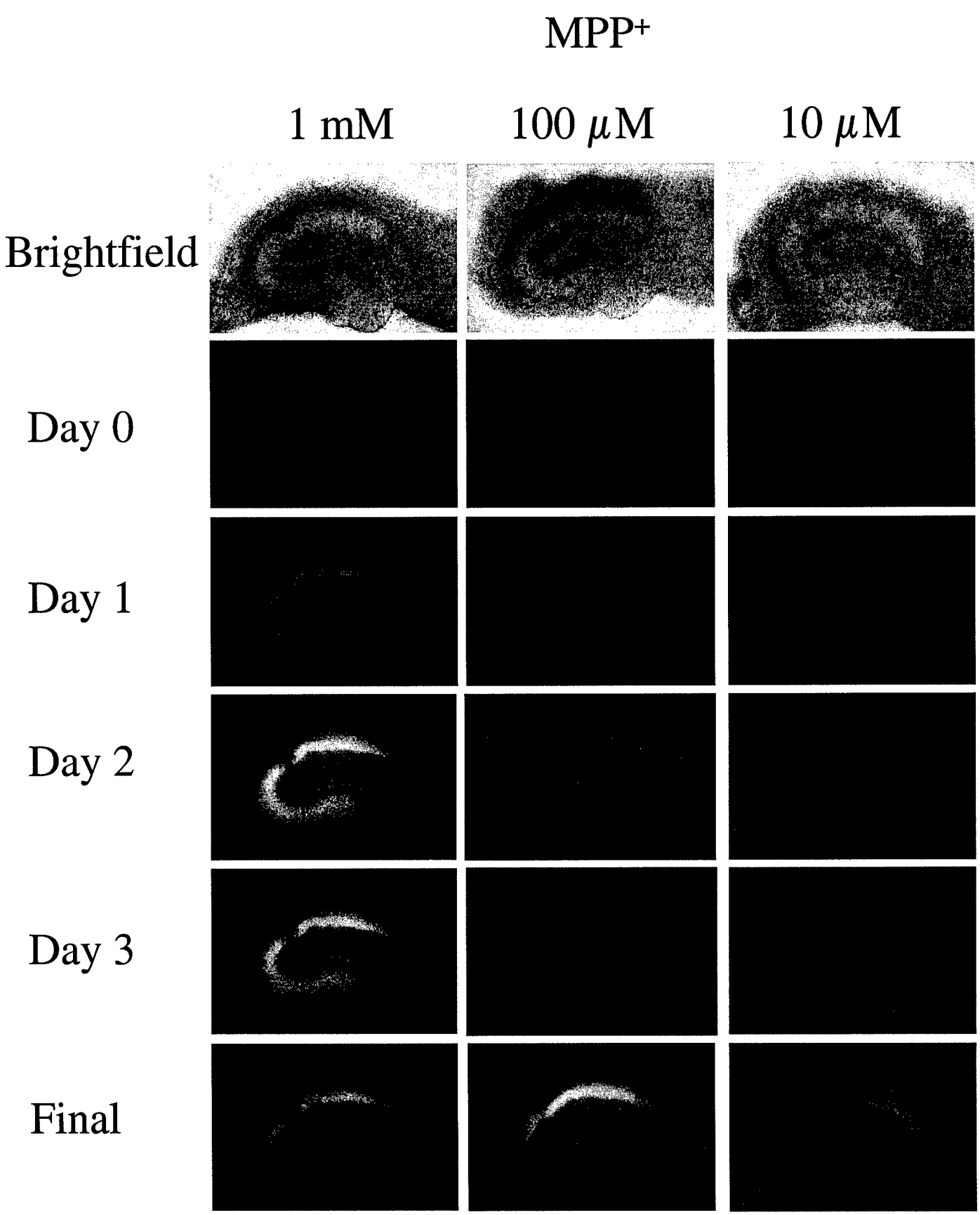


Figure 3

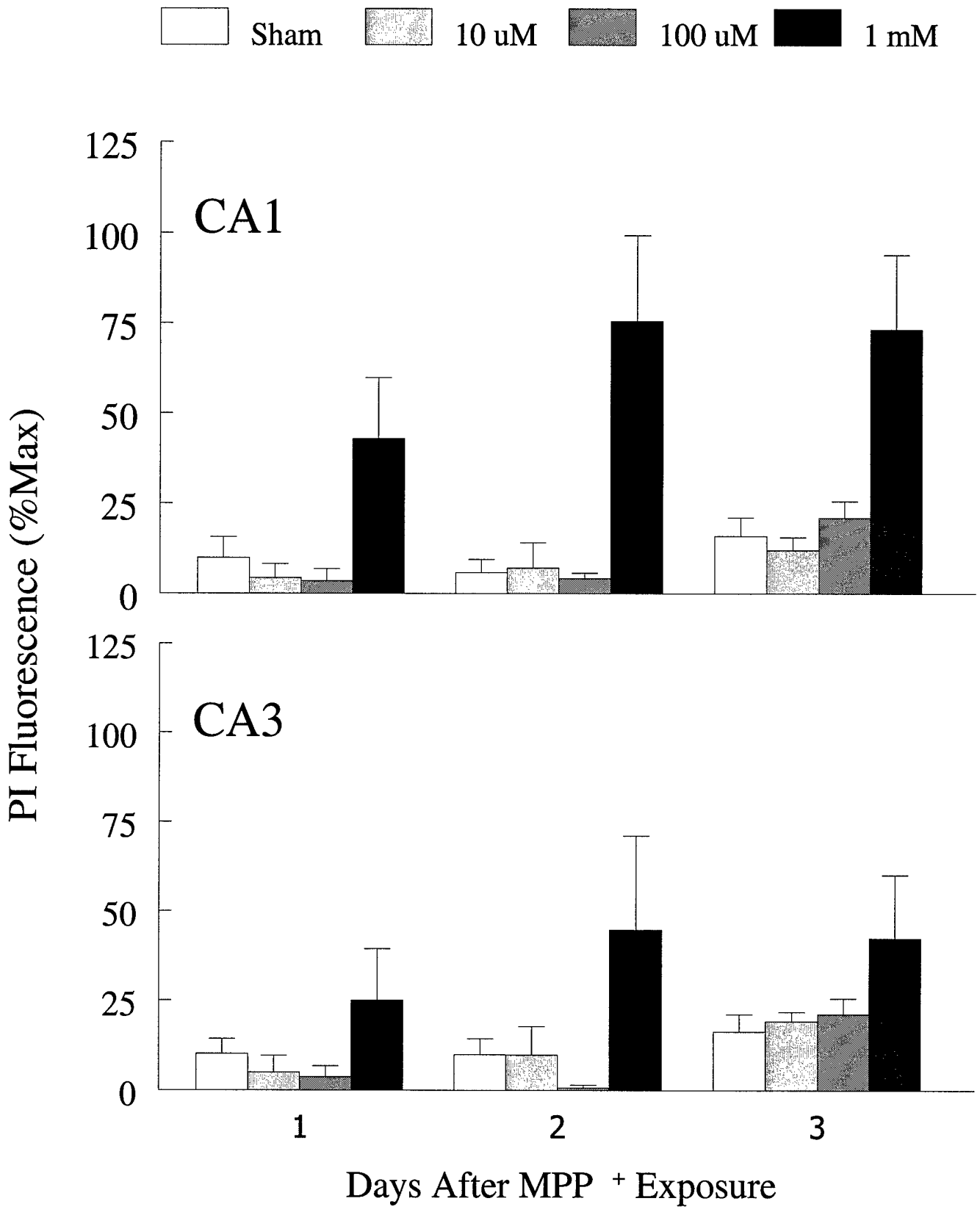
Sham
10 uM
100 uM
1 mM
CA1
CA3
PI Fluorescence (%Max)
125
100
75
50
25
0
1
2
3
Days After MPP + Exposure

Figure 4

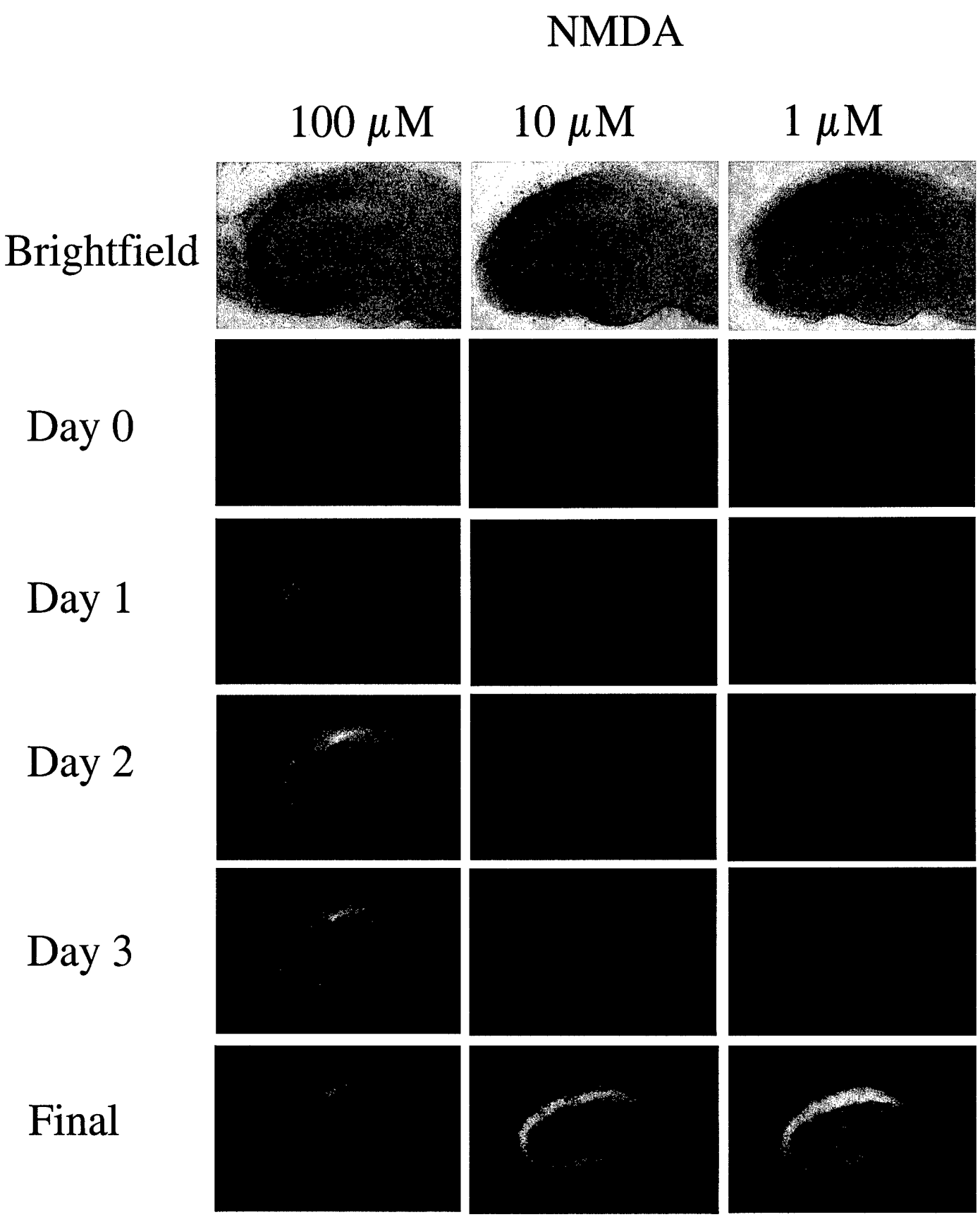


Figure 5

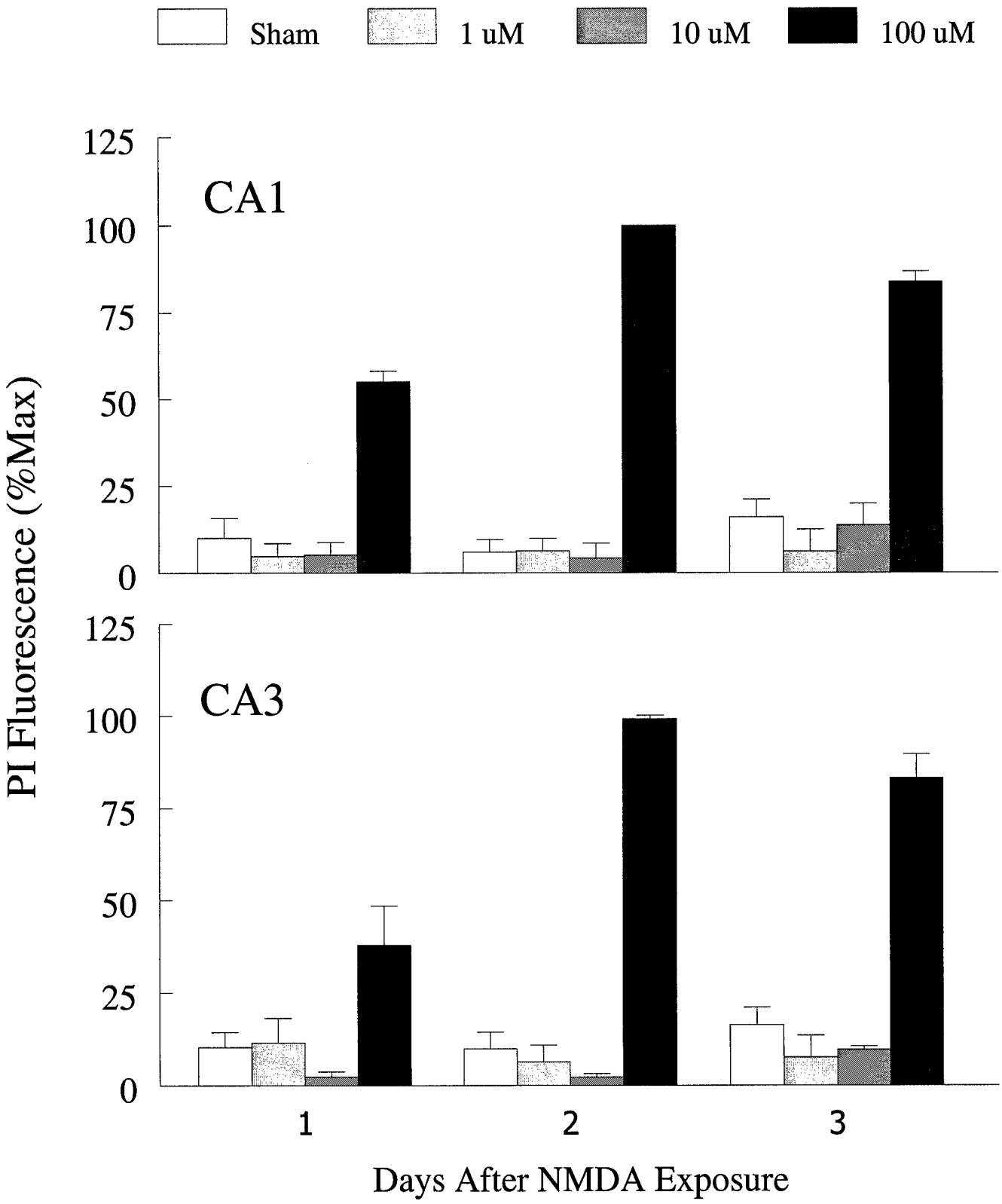
Sham
1 uM
10 uM
100 uM
CA1
CA3
PI Fluorescence (%Max)
125
100
75
50
25
0
1
2
3
Days After NMDA Exposure

Figure 6

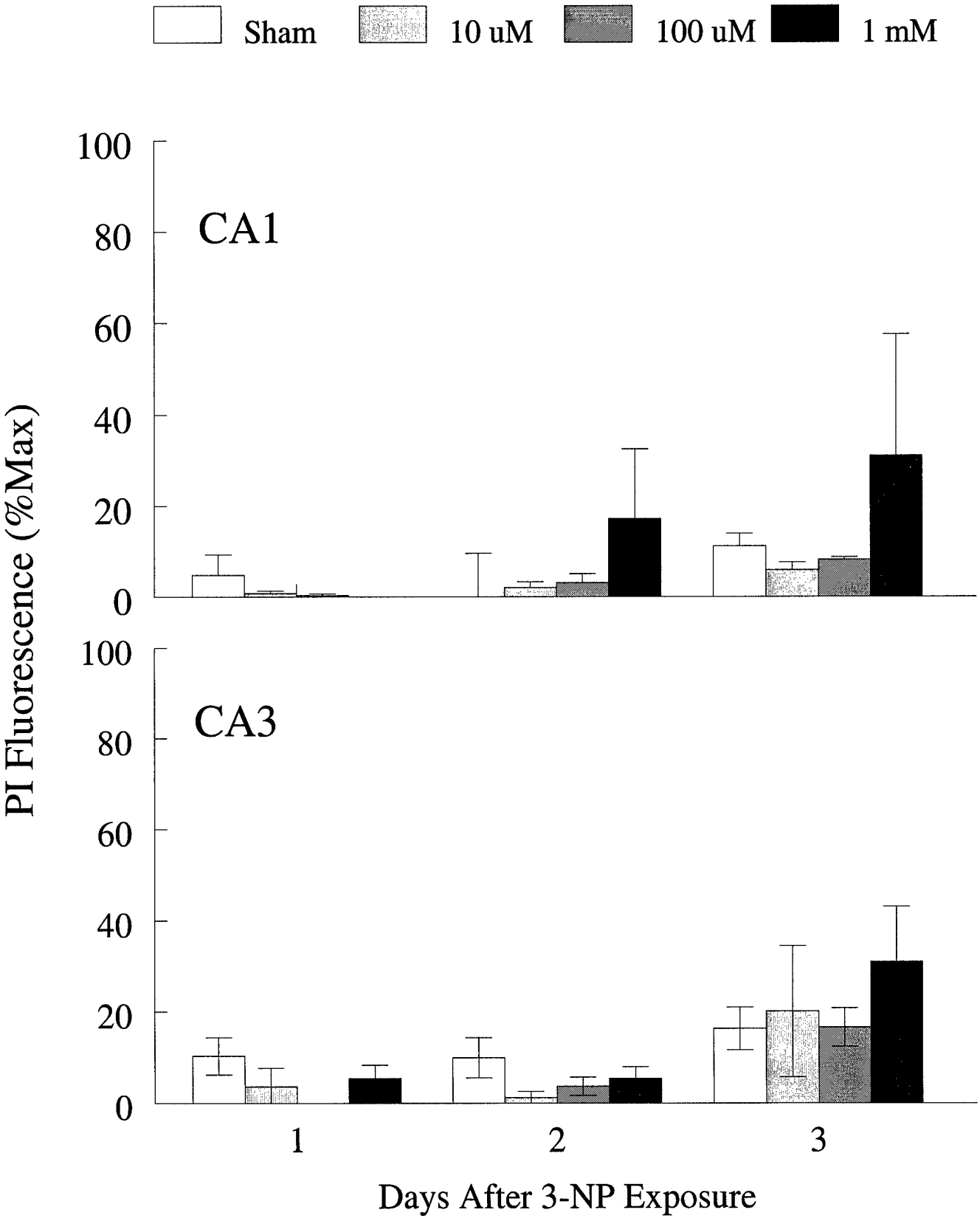
Sham
10 uM
100 uM
1 mM
CA1
CA3
PI Fluorescence (%Max)
100
80
60
40
20
0
1
2
3
Days After 3-NP Exposure

Figure 7